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Hydration of *Cuphea* seeds containing crystallised triacylglycerols

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Abstract. Seeds that exhibit intermediate storage behaviour seem to die under conventional −18°C storage conditions. *Cuphea wrightii* A. Gray, *C. laminuligera* Koehne, *C. carthagenensis* (Jacq.) J.F. Macbr. and *C. aequipetala* Cav are considered sensitive to low temperature storage. The seeds of these species have triacylglycerols (TAG) that are crystalline at −18°C and melt when the seeds are warmed to >35°C. In contrast, seeds of tolerant species, *C. lanceolata* W.T. Aiton and *C. hookeriana* Walp., have TAG that crystallise at temperatures below −18°C and are fluid at 22°C. *Cuphea* seeds imbided while TAG are crystalline fail to germinate and exhibit visual damage. However, germination proceeded normally when dry seeds were warmed adequately to melt any crystalline TAG before imbibition. Reduced germination and cellular disruption including loss of lipid body compartmentation and fragmented protein bodies develop in seeds with crystalline TAG equilibrated to >0.1 g H_2O g^{-1} DW. This damage cannot be reversed, even when seeds are dried before the damage can be visually detected. Results from this work reveal that the seeds of some species with intermediate type physiologies can be successfully placed into conventional −18 and −80°C storage facilities.

Additional keywords: intermediate storage behaviour, lipid, phase transition, seed, temperature, water.

Introduction

Seeds that survive desiccation, but do not survive long-term exposure to low temperatures have been termed 'intermediate' (Ellis *et al*. 1990). This type of seed physiology has not been well characterised, but has been described through case studies of individual species. *Azadirachta* (neem: Sacandé *et al*. 2000), *Citrus* (Hor *et al*. 2005), *Coffea* (coffee: Eira *et al*. 2006), and *Elaeis* (oil palm: Ellis *et al*. 1991) as well as 37 other genera have intermediate storage behaviour according to the IPGRI Electronic Seed Storage Behaviour (ESSB) Compendium (Hong *et al*. 1996). Seeds that exhibit intermediate storage behaviour have not traditionally been included in seed-based genebanks since they seem to die under conventional −18°C storage conditions.

Seeds of some *Cuphea* species exhibit storage behaviour consistent with intermediate physiology (Crane *et al*. 2003, 2006; Volk *et al*. 2006). Although tolerant of nearly complete desiccation, seeds containing triacylglycerols (TAG) with high levels of capric (C12) or caprylic (C14) acids do not germinate after storage at −18°C (Crane *et al*. 2003). High levels of saturated, medium-chain fatty acids cause TAG to crystallise at temperatures between 10 and −10°C, and subsequently melt at temperatures above 20°C (Crane *et al*. 2003, 2006; Volk *et al*. 2006). *Cuphea* seeds failed to germinate when they were cooled to temperatures that induced TAG crystallisation and then imbibed at temperatures that did not fully melt the TAG. However, germination proceeded normally when dry seeds were warmed to 45°C to ensure that all TAG returned to the fluid phase before imbibition (Crane *et al*. 2003, 2006; Volk *et al*. 2006). The same dynamic may be true for other intermediate seeds which contain TAG with high levels of saturated fatty acids and may, therefore, be prone to damage induced when water and crystallised TAG interact.

Storage and imbibition of seed containing TAG with high levels of medium (C8–C14) and long chain (>C14) saturated fatty acids may be problematic because phase changes occur at physiological temperatures and introduce the possibility of water and crystallised TAG interactions. Previously, we showed that the rate of TAG crystallisation in seeds of *C. carthagenensis*, which contain TAG that crystallise at 6°C, was dependent on seed moisture content, and that crystallisation-melting treatments did not reduce the viability of seeds containing ≤0.07 g H_2O g^{-1} DW (≤55% RH) (Crane *et al*. 2006). However, TAG crystallised within 3 days in *C. carthagenensis* seeds containing ≥0.13 g H_2O g^{-1} DW (≥75% RH) that were exposed to 5°C, and the seeds did not germinate following this treatment. These observations were consistent with previous observations that lethal interactions between crystallised TAG and water occurred during the earliest stages of seed imbibition (Crane *et al*. 2003).

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Cell damage during imbibition of seeds with crystallised TAG was previously visualised with transmission electron microscopy (TEM) (Volk *et al*. 2006). *C. wrightii* seeds, which contain TAG that crystallise at −8°C and melt between 22 and 39°C (transition peak at 31°C), were cooled to −18°C or −80°C and were imbibed at 22°C (i.e. TAG were crystalline), and TEM images from these seeds revealed cells with intact cell walls and plasmalemma, but disintegrated lipid bodies and sharp translucent shards penetrating through protein bodies, reminiscent of extant crystalline structures. Cellular disruption was less severe and took longer to appear in the cytoplasm of cold-exposed seeds that were imbibed at 5°C, even though this treatment was lethal to seeds of both *C. wrightii* and *C. lanceolata* [which contains TAG that crystallise at −20°C and melt between 12 and 28°C (transition peak at 19°C)] (Volk *et al*. 2006). The delayed expression and less severe symptoms in seeds imbibed at 5°C suggest that cell damage occurs in steps and might be reversible if seeds were re-dried before symptoms appeared and then re-imbibed after a temperature treatment to melt TAG.

The objectives of this paper were to determine (1) how much water is required to induce damage in seeds with crystallised TAG, (2) the point at which damage becomes apparent within cells during hydration, and (3) whether damage is reversible. We used six species of *Cuphea* that differ in their fatty acid composition, and therefore in their TAG crystallisation and melting temperatures. The lipid composition among *Cuphea* species is highly variable (Crane *et al*. 2003); but seeds of most species contain high levels of medium chain fatty acids and low levels (≤16%) of long chain fatty acids. We selected species with high levels of caprylic (C8) and capric (C10) acids (*C. lanceolata* and *C. hookeriana*), high levels of lauric (C12) acid (*C. laminuligera* and *C. carthagenensis*), high levels of capric (C10) and lauric (C12) acids (*C. wrightii*) and high levels of caprylic (C8) and myristic (C14) acids (*C. aequipetala*). The differences in fatty acid composition among *Cuphea* species allowed us to test for variable responses to low temperature and hydration treatments using congeneric seeds with a range of TAG melting temperatures (Table 1).

An understanding of the behaviour of seed lipids during storage and imbibition is important in the development of genebanking programs to conserve seeds that have traditionally been difficult to maintain for extended lengths of time. Optimisation of moisture contents will minimise the negative effects of crystalline TAG during seed storage or subsequent germination treatments.

Materials and methods

Plant materials and viability assessments

Seeds of *Cuphea wrightii* A. Gray (PI 594955), *C. lanceolata* W.T. Aiton (PI 594931), *C. aequipetala* Cav (PI 607971), and *C. carthagenensis* (Jacq.) J.F. Macbr. (Ames 17845) were greenhouse grown, harvested, and dried to ~0.05 g H_2O g^{-1} DW at the USDA-ARS North Central Regional Plant Introduction Station (NCRPIS) in Ames, IA, USA between 1999 and 2001. Seeds of *C. laminuligera* Koehne (PI 561483) and *C. hookeriana* Walp. (PI 534684) were processed by NCRPIS in 1992 and 1998, respectively. Seeds were stored at 4°C at NCRPIS. Seeds were sent to the USDA-ARS National Center for Genetic Resources Preservation (NCGRP) in Fort Collins, CO, USA in 1993 (*C. laminuligera*), 1999 (*C. hookeriana*) and 2003 (all other species), where they were stored at −18°C (*C. hookeriana* and *C. laminuligera*), 5°C (*C. aequipetala*) or 22°C (*C. carthagenensis*, *C. wrightii* and *C. lanceolata*). Earlier studies showed that seeds of *C. laminuligera* and *C. hookeriana* were and were not, respectively, sensitive to −18°C storage, but that damage to *C. laminuligera* could be reversed by warming the seeds to 45°C before they were imbibed (Crane *et al*. 2003). All seeds received a 1-h exposure to 45°C before use, to ensure that the TAG were fully melted at the onset of experiments (Crane *et al*. 2003).

Germination assays were used to assess viability of seeds receiving different moisture equilibration or imbibition treatments (see below). Two or three replicates of 25 seeds were placed on damp blotter paper in Petri dishes and incubated at 25°C with a 16/8-h light/dark cycle for up to 6 weeks. A seed was scored as germinated when both the radicle and hypocotyl emerged.

Table 1. Triacylglycerol composition in seeds of various *Cuphea* species

Seeds of *Cuphea* species either germinate (tolerant) or fail to germinate (sensitive) after 16 h exposure to −18°C. Fatty acid composition data for *Cuphea* species are from Crane *et al*. (2003). TAG melting temperatures for α, β′, β crystals are calculated as described by Crane *et al*. (2003) with standard values as described by Small (1988). Measured TAG crystallisation and melting temperatures were determined using differential scanning calorimetry. nd, not determined

		Fatty acid composition (%)					Calculated TAG Melting temperature (°C)			Measured temperature of TAG transitions Onset (°C)		Peak (°C)	
Species	Sensitivity to −18°C	Caprylic C8	Capric C10	Lauric C12	Myristic C14	≥C16	α	β′	β	Crystallisation	Melting	Crystallisation	Melting
Cuphea wrightii	Sensitive	nd	29	54	5	11	4	27	39	−8	22	−14	31
Cuphea laminuligera	Sensitive	nd	17	63	10	10	9	30	42	−1	30	−4	33
Cuphea carthagenensis	Sensitive		8	63	13	15	10	30	42	6	28	1	35
Cuphea aequipetala	Sensitive	25	1	2	56	16	19	24	40	−11	23	−13	31
Cuphea lanceolata	Tolerant	1	83	4	2	11	−13	17	30	−20	13	−26	17
Cuphea hookeriana	Tolerant	50	25	2	1	16	−3	−1	20	−44	−6	−51	−2

Measurement and calculation of TAG crystallisation and melting temperature

Thermal behaviour of the TAG in *Cuphea* seeds was measured with a differential scanning calorimeter (DSC7; Perkin-Elmer, Norwalk, CT, USA) as previously described (Crane *et al*. 2003). Seed samples weighing ~10 mg (3–20 seeds, depending on species) were cooled within the differential scanning calorimeter from 20 to −100°C at 10°C min^{-1} and then warmed to 50°C at the same rate. Crystallisation and melting transitions were observed as exothermic and endothermic events during cooling and warming, and the onset temperature and enthalpy of these phase changes were calculated with Perkin-Elmer software. TAG exhibit several crystalline structures (the most common forms are referred to as α, β′, and β) and standard thermal data for simple TAG (i.e. all three fatty acids are the same) are available (Small 1988). These standards were used to estimate melting temperatures for α, β′, and β crystals in TAG mixtures that are found in *Cuphea* seeds (Table 1). Estimates of TAG melting temperature were calculated from weighted averages based on fatty acid composition. The β′ melting temperatures used in the calculations are −21°C for caprylic acid, 18°C for capric acid, 35°C for lauric acid and 47°C for myristic acid.

Temperature, imbibition and moisture equilibration treatments

Dry seeds (0.05 g H_2O g^{-1} DW) were placed in a −80°C freezer overnight to induce crystallisation and then rewarmed to 5, 22 or 45°C to manipulate the TAG phase. The resulting TAG were either crystalline or fluid, depending on temperature and species. After the cooling–rewarming cycle, seeds were either placed on damp blotter paper at 5 or 22°C (imbibition treatments) or at different relative humidities at 5, 25 or 45°C (moisture equilibration treatments).

For imbibition treatments, seeds were cooled to −80°C and then either placed directly on damp blotter paper at 5 or 22°C or warmed to 45°C and then placed on damp blotter paper at 5 or 22°C. Seeds were imbibed for up to 4 h and then sampled for microscopy or transferred to germination conditions at 25°C for viability assessments. In another experiment, *C. wrightii* and *C. lanceolata* seeds were cooled to −80°C, imbibed at 5°C for 4 h, and then re-dried rapidly at 5°C. Seeds were then warmed to either 22 or 45°C for 1 h, imbibed at 22°C for 4 h, sampled for microscopy and then placed on damp blotter paper at 25°C to germinate.

For moisture equilibration treatments, relative humidity (RH) was controlled by saturated salt solutions in screw-cap jars. RH ranged from 32 to 93% depending on the temperature (5, 25, and 45°C) and salt: 34, 33, and 32% for $MgCl_2$; 61, 51, and 43% for $CaNO_3$; 72, 62, and 51% for NH_4NO_3; 76, 75, and 75% for NaCl; 88, 85, and 81% for KCl; and 93, 91, and 87% for KNO_3, respectively (Vertucci and Roos 1993). *C. wrightii* and *C. lanceolata* seeds were cooled to −80°C to induce TAG crystallisation and then placed in RH chambers at 5, 25 and 45°C, respectively, and allowed to equilibrate for 5 (5 and 25°C) or 3 days (45°C). After moisture equilibration, samples were split and seeds were placed at either 22°C or 45°C for 1 h, imbibed at 22°C for 4 h and then sampled for microscopy or germinated at 25°C.

Microscopy

Cellular structure was examined in seeds prepared as described above. Embryos (axes and cotyledons) excised from seeds were fixed overnight in 1.25% glutaraldehyde and 2% paraformaldehyde containing 0.05 M pipes buffer [piperazine-*N*,*N*-bis (2-ethanesulfonic acid)], post-fixed with 2% osmium tetroxide in 0.05 M pipes buffer, dehydrated in acetone, infiltrated with Spurr's resin (Electron Microscopy Sciences, Hatfield, PA, USA) and hardened overnight at 70°C (Volk *et al*. 2006). Thick sections (1 μm) were made with glass knives on a RMC MT-X ultramicrotome (Ventana Medical Systems Inc., Tucson, AZ, USA) and were stained with Stevenel's stain (del Cerro *et al*. 1980). Thin sections (70–90 nm) were cut with a diamond knife, mounted on formvar-coated copper grids (150 mesh) and stained with 3.2% uranyl acetate in 50% methanol and 50% ethanol for 15 min and Reynold's lead citrate (Bozzola and Russell 1991) for 15 min.

Evidence of cellular damage was visualised in thick sections with light microscopy or in thin sections with a JEOL 2000 EXII transmission electron microscope (TEM; Jeol Ltd., Tokyo, Japan) at 100 kV accelerating voltage (Volk *et al*. 2006). The percentage of disrupted cells was calculated with images obtained from the light microscope for three regions containing between 8 and 20 cells from at least three seeds for each treatment. Each experiment was repeated once and data from replicate experiments were combined.

Results

TAG transition temperatures and germination response in diverse Cuphea *species*

Triacylglycerols extracted from seeds of *Cuphea* species varied in proportions of caprylic (C8), capric (C10), lauric (C12) and myristic (C14) acids (Table 1). Melting transitions for simple TAG comprised of these fatty acids range from <−15 to 33°C (α), −21 to 46.5°C (β′) and 8.3 to 57°C (β), depending on fatty acid chain length (Small 1988). Seed TAG melting transitions were calculated by weighted averages of the known fatty acid composition of *Cuphea* species (Crane *et al*. 2003). Melting temperatures calculated for β′ crystals corresponded well to those measured by differential scanning calorimetry (scans are not provided, but melting transition temperatures are summarised in Table 1) (Crane *et al*. 2003). The temperature at which the TAG melt varied widely among species. TAG of *C. hookeriana* seeds, with an endotherm peak of −2°C, were fluid when warmed to only 5°C, and warming to 22°C will melt crystalline TAG in *C. lanceolata* seeds (temperature of endotherm peak is 17°C). However, warming to temperatures above 35°C was required to melt the crystalline TAG of *C. wrightii*, *C. laminuligera*, *C. carthagenensis*, and *C. aequipetala*, for which the temperature of endotherm peaks were 31°C or higher (Table 1). TAG crystallisation temperatures were also dependent on fatty acid composition and were as much as 50°C lower than the TAG melting temperature for individual species. Therefore, although a −18°C exposure was sufficient to induce crystallisation in seeds of species deemed sensitive to low temperature (*C. wrightii*, *C. laminuligera*, *C. carthagenensis*, and *C. aequipetala*), a −80°C exposure was required to induce crystallisation in all six species used in this study.

Results from viability tests on seeds given various temperature treatments before imbibition support the hypothesis that hydration of seeds containing crystalline TAG is lethal. For all species studied, control samples that did not receive a −80°C treatment had high germination percentages when imbibed at 22°C. High levels of germination were also achieved following a −80°C exposure for all samples that received a subsequent 45°C treatment before imbibition at 22°C (Table 2). Low temperature treated *C. lanceolata* and *C. hookeriana* seeds had high germination rates when warmed and imbibed at 22°C. However, this same temperature cycle (−80 to 22°C) was lethal to seeds of *C. wrightii*, *C. laminuligera*, *C. carthagenensis*, and *C. aequipetala.* In these species, the −80 to 22°C temperature cycle maintained TAG in a crystalline state. Viability was restored when these seeds were heated to 45°C such that seed TAG were in a fluid state before imbibition at 22°C (i.e. temperature cycle was −80 to 45 to 22°C; Table 2).

Cell damage after imbibition of seeds containing crystalline TAG was visualised by microscopy. Germination was inversely proportional to the percentage of cells displaying visual damage. Seeds capable of germination had cells with spherical protein and lipid bodies contained by visible membranes (Fig. 1*A*). TEM of cells from *C. wrightii*, *C. laminuligera*, and *C. carthagenensis* seeds imbibed while TAG were crystalline showed intact cell walls but dramatic evidence of cellular disruption: membranes that once surrounded lipid bodies were absent and remnants of protein bodies were dispersed into smaller, irregular shapes with electron lucent intrusions, similar to those pictured in Fig. 1*C–F*. No germination was recorded for seeds of *C. aequipetala* imbibed while TAG were crystalline, attesting to severe damage. However, cells of seeds given this treatment only exhibited common symptoms of cellular degradation: absence of cellular compartmentalisation and dispersed cytoplasm similar to those depicted in Fig. 2*B*, *D*.

Development of visible symptoms of cell damage

The timing of cellular disruption was evaluated in *C. wrightii* seeds that were exposed to −80°C and then warmed to 5°C or room temperature and imbibed for 0–4 h before fixation at 22°C. After 0.5 h of imbibition at 22°C, cellular disruption was not detected with light microscopy (data not shown), but evidence of damage, protein body intrusions, was detected with TEM (Fig. 1*B*). Cellular disruption was readily apparent from tissues of seeds imbibed for 1 and 2 h at 22°C with both light microscopy (data not shown) and TEM (Fig. 1*D*, *F*), with increasingly dispersed protein bodies observed at the 2-h timepoint. Loss of cellular integrity in *C. wrightii* seeds was apparent when seeds that were initially imbibed at 5°C were transferred to room temperature and fixed after 0 or 0.5 h (Fig. 1*C*, *E*).

Effect of moisture content on viability and cell damage

This part of the study, using seeds of *C. wrightii* and *C. lanceolata*, was designed to ascertain how much water was needed to induce lethal symptoms in seeds with crystalline TAG. TAG within dry seeds of both species were crystallised by a −80°C treatment. Seed moisture content was then adjusted by exposure to different RH at 5°C (TAG remained crystallised in both species), 25°C (TAG were fluid in *C. lanceolata* but not in *C. wrightii*), or 45°C (TAG were fluid in both species). By treating half of the moisture-equilibrated seeds at 45°C before imbibition at 22°C, we were able to separate the effects of moisture equilibration from those described previously for imbibition.

Table 2. Viability and cellular disruption assessments for various *Cuphea* species

Germination data are provided for seeds of *Cuphea* species that were not treated, were cooled to −80°C, or were cooled to −80°C and then were warmed to 45°C before a 22°C imbibition. The percentage of cells exhibiting cellular disruption resulting from hydrating crystalline TAG is recorded

Species	Exposure temperature for dry seeds		Lipid phase	Viability assessment	
	Cooled to (°C)	Warmed to (°C)	during imbibition	Germination (%)	Cellular disruption (%)[A]
Cuphea wrightii	None	None	Fluid	71 ± 5	10 ± 10
	−80	22	Crystalline	0	100 ± 0
	−80	45	Fluid	70 ± 2	0
Cuphea laminuligera	None	None	Fluid	65 ± 4	31 ± 19
	−80	22	Crystalline	0	61 ± 17
	−80	45	Fluid	59 ± 6	3 ± 3
Cuphea carthagenensis	None	None	Fluid	77 ± 8	18 ± 12
	−80	22	Crystalline	0	38 ± 19
	−80	45	Fluid	79 ± 2	18 ± 18
Cuphea aequipetala	None	None	Fluid	80 ± 8	0[B]
	−80	22	Crystalline	0	0[B]
	−80	45	Fluid	70 ± 4	0[B]
Cuphea lanceolata	None	None	Fluid	84 ± 5	0
	−80	22	Fluid	93 ± 4	0
	−80	45	Fluid	90 ± 5	0
Cuphea hookeriana	None	None	Fluid	75 ± 5	0
	−80	22	Fluid	82 ± 7	0
	−80	45	Fluid	82 ± 7	0

[A]Damage resulted from hydrating crystalline TAG.

[B]Cells appeared degraded rather than disrupted as a result of hydrating crystalline TAG.

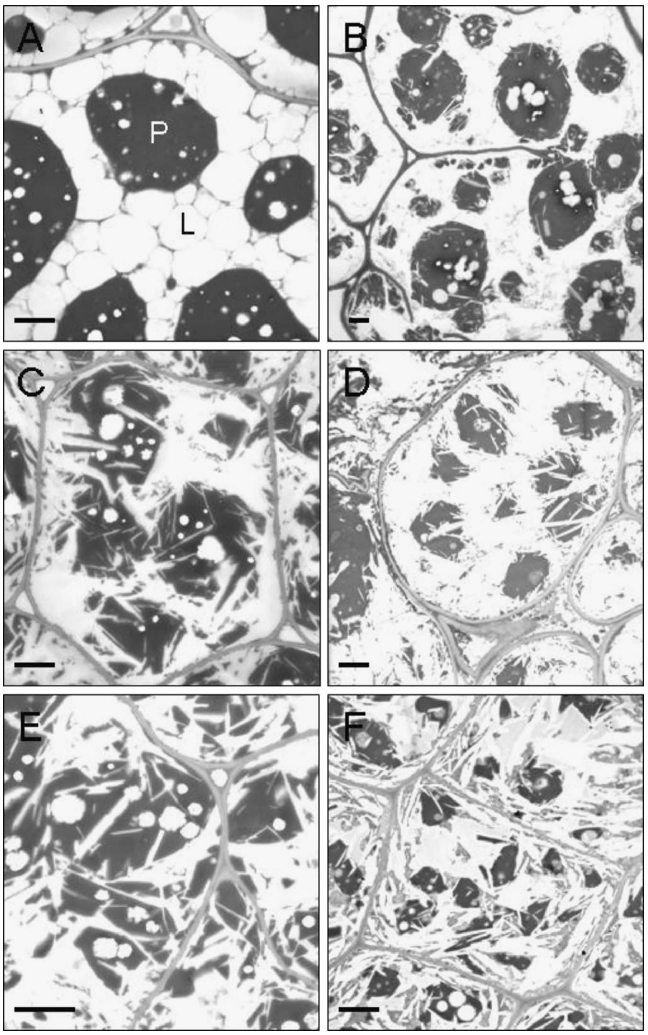

Fig. 1. Ultrastructure of cotyledon cells from *Cuphea wrightii* seeds after exposure to −80°C and either immediately imbibed at 22°C for 0 (*A*), 0.5 (*B*), 1 (*D*) or 2 h (*F*) or first imbibed at 5°C for 4 h and then imbibed at 22°C for 0 (*C*) or 0.5 (*E*) before chemically fixing seed tissues at 22°C. Cellular disruption is evidenced by the loss of lipid body compartmentation and the presence of protein bodies with electron lucent protrusions. P, protein body; L, lipid body. Scale bar = 2 µm.

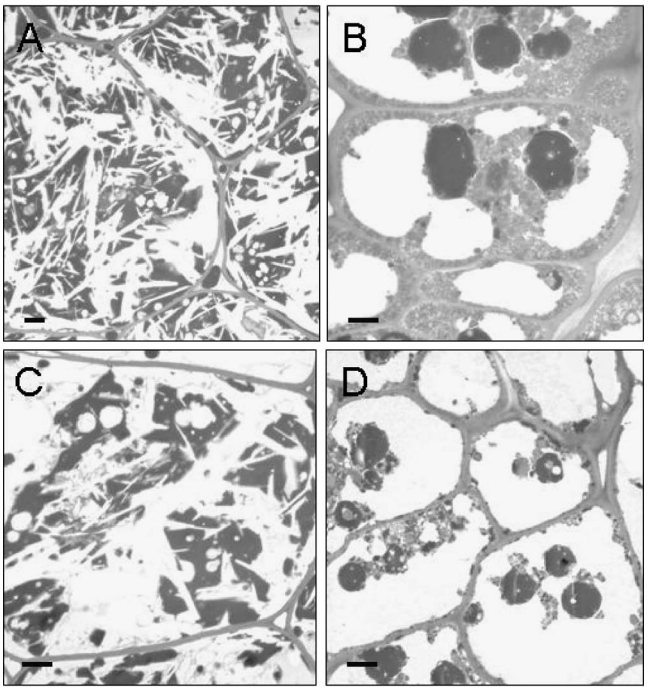

Fig. 2. Ultrastructure of cotyledon cells from *Cuphea wrightii* (*A*, *C*) and *Cuphea lanceolata* (*B*, *D*) that were first imbibed at 5°C for 4 h, then re-dried to 0.06 g H_2O g^{-1} DW and imbibed at 22°C for 4 h directly after desiccation (*A*, *B*) or imbibed after seeds were treated at 45°C for 2 h (*C*, *D*). Cellular disruption (described in Fig. 1) is apparent for *C. wrightii* (*A*, *C*). In *C. lanceolata*, cellular degradation as evidenced by lack of visible lipid bodies, indistinct margins around the protein bodies and a lack of definition of the cytoplasm and organelles (*B*, *D*). Scale bar = 2 µm.

Relative humidities between 34 and 43% did not significantly increase seed moisture content (Table 3). When RH was greater than 51%, seed moisture content increased to between 0.07 and 0.27 g H_2O g^{-1} DW. *C. wrightii* seeds that were moisture equilibrated at 5 or 25°C and then imbibed at 22°C without the 45°C treatment failed to germinate. Keeping seed moisture content less than 0.10 g H_2O g^{-1} DW while TAG were in the crystalline state (5°C for *C. lanceolata* and ≤25°C for *C. wrightiii*) and then treating seeds at 45°C before imbibition resulted in >50% germination (Table 3). Germination percentages progressively decreased when the equilibrated moisture content exceeded 0.10 g H_2O g^{-1} DW in seeds that were equilibrated with TAG in the crystalline state and then treated at 45°C before imbibition. *C. lanceolata* seeds that had low germination without the 45°C heat pulse had moisture contents of ≥0.17 g H_2O g^{-1} DW at 5°C and ≥0.14 g H_2O g^{-1} DW at 45°C.

Seeds of *C. wrightii* that were imbibed under conditions that maintained crystalline TAG exhibited cellular disruption (loss of lipid bodies, protein bodies with electron lucent protrusions) (similar to cells observed in Fig. 1*C–F*). Cellular disruption was also evident in *C. wrightii* seeds that were equilibrated to moisture contents of ≥0.12 g H_2O g^{-1} DW at both 5°C and 25°C and then imbibed after the 45°C heat pulse. Damage was minimal when *C. wrightii* seeds were equilibrated at 45°C. In contrast, *C. lanceolata* seeds that were equilibrated to moisture contents of ≥0.17 g H_2O g^{-1} DW and then imbibed either with or without the 45°C heat pulse had cellular degradation resembling that described above for *C. aequipetala*. *C. lanceolata* seeds equilibrated to lower moisture contents at 5°C or at any moisture content at 25 or 45°C did not have damage that could be visualised with the light microscope (Table 3).

Low germination percentages were also obtained for seeds of both *C. wrightii* and *C. lanceolata* equilibrated at 45°C and >80% RH. This treatment resembles the high humidity, high temperature conditions used to achieve accelerated aging in seeds, resulting in a low germination percentage (McDonald 1999).

Table 3. Germination and cellular damage assessments for *Cuphea wrightii* and *Cuphea lanceolata* seeds equilibrated to different relative humidities before imbibition

Seeds were equilibrated to different relative humidities by placing them in chambers over saturated salt solutions at 5, 25 or 45°C. Seed moisture content, germination, and cellular disruption assessments were obtained for both *C. wrightii* and *C. lanceolata* when seeds were imbibed immediately after moisture equilibration or after a 1-h 45°C treatment before imbibition at 22°C

				Cuphea wrightii				*Cuphea lanceolata*			
Equilibration temperature	TAG phase	Relative humidity (%)	Seed water content ($g H_2O g^{-1}$ DM)	+22°C only Germination (%)	+22°C only Cellular disruption (%)	+45°C for 1 h then 22°C Germination (%)	+45°C for 1 h then 22°C Cellular disruption[A] (%)	+22°C only Germination (%)	+22°C only Cellular disruption[A] (%)	+45°C for 1 h then 22°C Germination (%)	+45°C for 1 h then 22°C Cellular disruption[A] (%)
5°C	All TAG	34	0.05	0	–	49 ± 6	–	82 ± 13	–	91 ± 7	–
	crystalline	61	0.09	0	–	54 ± 10	–	72 ± 14	–	93 ± 5	–
		72	0.12	0	67 ± 15	45 ± 10	13 ± 8	55 ± 28	0 ± 0	86 ± 6	0 ± 0
		76	0.13	0	33 ± 33	35 ± 21	0 ± 0	71 ± 20	0 ± 0	89 ± 8	0 ± 0
		88	0.17	0	40 ± 25	14 ± 8	18 ± 12	47 ± 25	0 ± 0[B]	64 ± 2	0 ± 0[B]
		93	0.27	0	86 ± 12	3 ± 2	11 ± 5	20 ± 14	0 ± 0[B]	26 ± 18	0 ± 0[B]
25°C	Crystalline	33	0.06	0	–	73 ± 1	52 ± 2	89 ± 5	–	96 ± 3	0 ± 0
	in *Cuphea wrightii*	51	0.07	0	–	76 ± 5	–	89 ± 1	–	96 ± 1	0 ± 0
	and fluid in	62	0.09	0	–	76 ± 6	15 ± 15	94 ± 4	–	90 ± 1	0 ± 0
	Cuphea lanceolata	75	0.13	0	–	26 ± 1	33 ± 14	92 ± 5	–	85 ± 1	0 ± 0
		85	0.17	0	–	0	37 ± 22	95 ± 1	–	81 ± 11	0 ± 0
		91	0.19	0	–	0	35 ± 18	86 ± 1	–	84 ± 2	0 ± 0
45°C	All TAG fluid	32	0.05	81 ± 4	–	69 ± 5	–	96 ± 1	–	90 ± 1	–
		43	0.05	81 ± 2	–	79 ± 1	–	92 ± 1	–	97 ± 2	–
		51	0.07	73 ± 3	–	66 ± 3	0 ± 0	82 ± 1	–	94 ± 2	0 ± 0
		75	0.11	65 ± 11	–	48 ± 5	2 ± 2	74 ± 8	–	72 ± 4	0 ± 0
		81	0.14	39 ± 1	–	44 ± 6	0 ± 0	49 ± 1	–	32 ± 1	0 ± 0
		87	0.18	2 ± 2	–	5 ± 1	0 ± 0	34 ± 5	–	32 ± 1	0 ± 0

[A]Cellular disruption resulted from hydrating crystalline TAG.

[B]Cells appeared degraded rather than disrupted.

Hydration-dehydration cycles

Cuphea wrightii seeds exposed to −80°C, imbibed at 22°C or 5°C for 4 h and then re-dried failed to germinate whether or not they received a 45°C heat pulse treatment before they were reimbibed (Table 4). The initial 4-h imbibition was not sufficient to induce visible cellular disruption when *C. wrightii* was imbibed at 5°C (Volk *et al*. 2006). However, cells in seeds that were re-dried and then reimbibed at 22°C for 4 h exhibited massive cellular disruption similar to that observed during the first imbibition treatment when seeds with crystalline TAG were hydrated (compare Fig. 2*A* with Fig. 1*F*).

Cuphea lanceolata seeds exposed to −80°C, imbibed with TAG in the fluid state at 22°C, desiccated, and then imbibed again at 22°C had high germination either with or without the 45°C treatment (Table 4). A 4-h imbibition did not result in visible cellular changes when seeds of *C. lanceolata* were imbibed at 5°C (TAG in the crystalline state) even though this treatment is lethal (Crane *et al*. 2003; Volk *et al*. 2006). However, seeds did not germinate when they were imbibed at 5°C for 4 h, then desiccated and then imbibed a second time at 22°C even with and without the 45°C heat pulse (Table 4). Although cellular disruption resulting from the first hydration of crystalline TAG at 5°C was not observed in *C. lanceolata* seeds, cells observed from both the 22 and 45°C re-imbibition treatments did not have visible lipid bodies. Even though the protein bodies in these *C. lanceolata* cells appeared somewhat intact, the cytoplasm and organelles lacked definition (Fig. 2*B*, *D*).

Discussion

The lipid composition of seeds in the *Cuphea* genus varies widely. Some species tolerate cold temperature exposure since they have TAG that melt when seeds are brought to standard imbibition and germination conditions. Other species containing TAG with higher melting temperatures only germinate when seeds are warmed to temperatures above 35°C following low temperature exposure. Regardless of lipid composition, seeds of the *Cuphea* species studied fail to germinate when they are hydrated while their TAG are in a crystalline state.

The seed fatty acid composition of *Cuphea* species determines sensitivity to hydration following low temperature exposure. *C. wrightii*, *C. laminuligera* and *C. carthagenensis* have TAG melting temperatures between 22 and 30°C (Table 1). The primary (>50%) fatty acids of the seeds of these species are comprised of lauric acid (β′ melting temperature 35°C). The presence of capric acid in *C. wrightii* (29%) appears to lower the TAG melting temperature. The seeds of *C. laminuligera* and *C. carthagenensis* both contain capric and myristic acid (Table 1). Capric acid (β′ melting temperature 18°C) lowers and myristic acid (β′ melting temperature 47°C) raises the seed TAG melting temperatures. When *C. wrightii*, *C. laminuligera* and *C. carthagenensis* seeds are hydrated under conditions that maintain crystalline TAG, cellular disruption (loss of lipid body compartmentation and the presence of protein body intrusions) is observed and the seeds fail to germinate.

Cuphea aequipetala seeds have a TAG melting temperature of 23°C. TAG in these seeds are composed of 56% myristic acid and 25% caprylic acid (β′ melting temperature −21°C). *C. aequipetala* failed to germinate after low temperature exposure and subsequent hydration. The combination of both the high and low melting temperature fatty acids within the seeds may have caused the cellular degradation that was visualised as a result of crystalline TAG damage. *C. lanceolata* and *C. hookeriana* both withstand low temperature exposure and imbibition at 22°C. They have TAG melting temperatures of 13 and −6°C (Table 1) resulting from high levels of caprylic and capric fatty acids (β′ melting temperatures of −21 and 18°C, respectively).

Cold sensitive *Cuphea* species that have been exposed to temperatures that result in TAG crystallisation do not germinate when the calculated β′ melting temperature is above the temperature at which they are imbibed. There is a good correspondence between the predicted β′ melting temperatures and the actual TAG melting temperatures determined by differential scanning calorimetry (Table 1). Calculated β′ melting temperatures should be considered when establishing storage or germination protocols for species with intermediate type seed storage physiologies.

The development of cellular damage during imbibition was compared with loss of germination. Cellular disruption appeared within 0.5 h when cold sensitive, cold exposed *Cuphea* seeds were imbibed at 22°C (Fig. 1*B*). Loss of viability was immediate: seeds given similar temperature treatment and hydrated to 0.10 g $H_2O\,g^{-1}$ DW at 22°C failed to germinate (Table 3). Cellular damage was not immediately apparent in cold-exposed seeds imbibed at 5°C. Cells of cold exposed *C. wrightii* seeds appeared intact but degraded after 24 h imbibition at 5°C and fixation at the same temperature (Volk *et al*. 2006). Massive cellular disruption became immediately obvious if 5°C imbibed seeds were transferred to 22°C for fixation (Fig. 1*C*). The slower appearance of cellular damage during 5°C imbibition suggested that there was less damage during initial imbibition and that it

Table 4. Germination percentages for *Cuphea* seeds exposed to −80°C, imbibed and then re-dried before a second imbibition

Seeds of *Cuphea wrightii* and *Cuphea lanceolata* were imbibed for 4 h at the indicated temperature and then desiccated to 0.06 g $H_2O\,g^{-1}$ DW. Half of the seed treatments were then exposed to a 45°C treatment for 1 h before a second imbibition at 22°C

Temperature (°C) during 1st imbibition	Desiccation following imbibition	45°C Pulse (1 h) after desiccation	Temperature during 2nd imbibition	Germination %	
				Cuphea wrightii	*Cuphea lanceolata*
22	Yes	No	22	0	83 ± 5
22	Yes	Yes	22	0	89 ± 7
5	Yes	No	22	0	0
5	Yes	Yes	22	0	0
5	No	No	22	0	0

could possibly be reversed. However, electron micrographs of re-dried seeds clearly show that although cellular damage was not visualised during initial imbibition at 5°C, it had occurred and was irreversible (Fig. 2*A–D*). Germination data support this conclusion because seeds imbibed briefly at 5°C and re-dried failed to germinate (Table 4) and germination percentages were low in seeds exposed to RH > 75% at 5°C (Table 3). Our data suggest that the seed deaths and cellular disintegration resulted from perturbations in cell structures that occurred during the earliest stages of hydration but could not be visualised using the current techniques.

Cuphea wrightii seeds exhibit cellular disruption and fail to germinate when equilibrated at high RH and low temperatures (5°C) (Table 3). The quantity of water absorbed by seeds at high RH is enough to induce the physiological response that results in reduced germination. Our results are consistent with earlier findings that *C. carthagenensis* seeds hydrated to moisture contents of greater than $0.12\,g\ H_2O\,g^{-1}$ DW have less than 50% germination when stored for 30 days or less at 5°C, even when treated with a 45°C pulse before imbibition and germination (Crane *et al*. 2006). High humidity and high temperature equilibration conditions are also not favourable for maintaining high quality seeds and result in cellular degradation as described in the literature (Table 3; McDonald 1999). Viability progressively decreases as relative humidity and seed water content increase under low temperature conditions.

This study demonstrated a lethal interaction between water and TAG that is regulated by temperature. We do not understand the basis of this interaction, but suggest that it involves changes in polar–non-polar partitioning with hydration (e.g. Golovina and Hoekstra 2002). We speculate that the interactions occur at the oilbody interface and lead to displacement of the hydrophobic core of oleosins (Herman 1995) when TAG crystallise. Although there is no specific precedence for this in the literature, it is well known that crystallisation leads to repartitioning and phase separation (Hoekstra *et al*. 2001).

Although water and lipid do not mix, we have shown a profound interaction between water and crystalline TAG in seeds. Brief imbibition or exposure of seeds to >75% RH is lethal and causes massive cellular disruption in seeds containing crystalline TAG. The incidence of damage depends on the melting temperature of TAG. Thus, seeds containing TAG with long or medium chain saturated fatty acids have high melting temperatures and are predisposed to this type of damage. The interaction between crystalline TAG and water may be the mechanism behind so-called intermediate storage physiology. Seeds with this physiology are not currently genebanked because the storage conditions are lethal. However, our study shows that they can survive conventional storage if they are warmed before planting.

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